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Abstract

The reversed-phase chromatography of small proteins and peptides at low pH with mobile phases modified with large proportions of organic solvents has become routine. Retention in these systems correlates well with values predicted from amino acid composition. A model of the sorption of proteins to modified silicas provides information that is extending the utility of HPLC into areas previously tractable only by the slower, more traditional chromatography. Measured surface tensions of proteins (γ_{pv}) are generally in the 65-70 ergs/cm^2 range, whereas typical surface tensions of column packings (γ_{sv}) may range from 30-50 ergs/cm^2, depending on the structure of the bound groups. Examination of the interrelationships of γ_{sv}, γ_{pv}, and γ_{mv} (γ_{mv} mobile phase surface tension) reveals that desorption of protein is increased by reducing γ_{mv}. This is achieved through addition of organic modifiers to the buffer. Desorption may also be increased by an increase in γ_{sv}. This is achieved through syntheses that produce surfaces that are essentially hydrophilic but lightly loaded with alkyl groups. Multisite interactions also control retention of proteins in ion-exchange chromatography. Slight changes in pH or ionic strength of mobile phase exert dramatic effects on retention volume. An understanding of these factors in the control of sorption/desorption along with advances in affinity chromatography provide a battery of techniques for faster and more selective protein separations. Use of efficient post-column reactors increase sensitivity and selectivity of detection. Automation and use of modified silicas greatly facilitate the task of sample preparation.

Introduction

High Performance Liquid Chromatography (HPLC) has taken its place with electrophoretic and other columnar methods for the analysis, isolation, and characterization of proteins and other biopolymers. International meetings are devoted to the subject, and major portions of most HPLC meetings are comprised of papers on protein and peptide methodology. For a long time, separations scientists had difficulty coming to grips with the fact that "proteins" were a class of widely dissimilar molecules and could not be viewed simply as a homologous series.

Polypeptides and small proteins have proved to be quite amenable to separation by HPLC but problems still remain if HPLC is to be applied to large proteins in an efficient and cost effective manner. Among these problems are column stability, separation selectivity, protein recovery and detection. Solutions are evolving steadily through a greater understanding of factors influencing retention and mass transport as well as the development of on-line, specific assay techniques. This chapter will describe the status of research that is leading to new developments in protein chromatography.

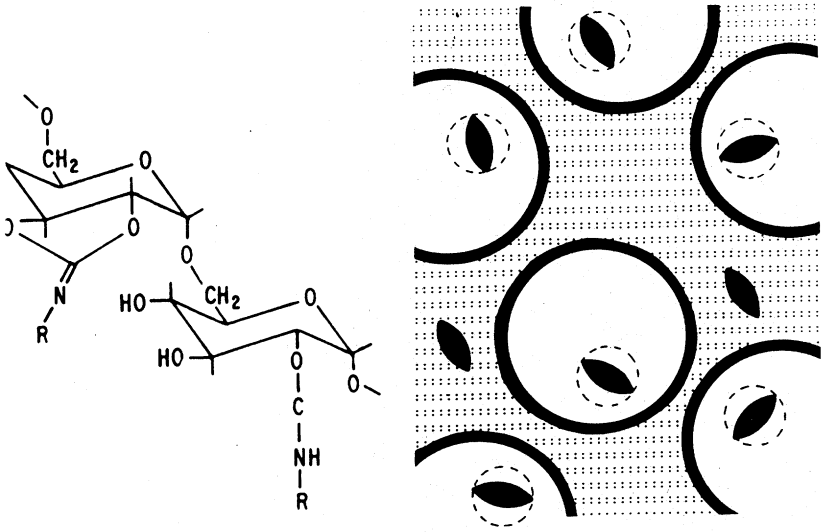
Theoretical and Applications

Traditional vs. high performance chromatography.

The gel-like packings that biochemists have long employed for enzyme isolations and characterizations, as well as the chromatographs, contrast in several ways to the high performance systems.

First, the traditional gel materials consist of loosely woven strands of carbohydrate polymer. More rigidity has been imparted into packed beds of the gels through introduction of cross-links between strands of a single fiber (1). The carbohydrate matrix may be modified chemically to produce such functional groups as epoxy, imidocarbonate, or carboxyl that, in turn, serve as reactive sites to which other moieties are attached. Thus, packings with ion-exchange groups, hydrophobic arms, lectins, or enzymes can be synthesized. Typically, the amount of active group is approximately 15 μg/ml bed volume, thus the interaction sites are widely spaced.

Columns packed with 3-5 micron–sized silica-based packings are quite different. The bed is tightly packed and the surface concentration of functional groups is on the order of meq/ml bed volume. Thus, from spacial considerations, while interactions between protein and functional groups on gel matrices involve only a few sites, a large number



1. Schematic comparison of packing surfaces. (a) Carbohydrate gel; (b) silica. l dots indicate surface groups; ellipses are protein.

tes are involved in interactions with siliceous materials. The two ·s of packings are represented schematically in Figure 7.1. Silicas ? about four silanol groups per hundred square angstroms of sur- (small dots in Figure 7.1.b). Protein molecules, therefore, would ·lap with hundreds of groups (ellipses in Figure 7.1.b). Considering diverse nature of surface groups of proteins, sorption is expected .rdless of type of group on the silica.

tein adsorption

)tion of proteins onto low energy surfaces such as modified silicas · be considered in terms of Van der Waal interactions between pro- and surface across a film of mobile phase. Van der Waal interac- s consist of forces between: (a) permanent dipoles, (b) dipoles ıced by permanent dipoles and (c) statistical dipoles resulting from lom motion of electrons. The Helmholz energy of interaction is given ·quation 1: $\Delta F_{smp} = \gamma_{sp} - \gamma_{sm} - \gamma_{mp}$, where γ is the interfacial tension and subscripts s, m, p designate column support, mobile phase and pro- , respectively (2). The interfacial tensions may be calculated from ·rimentally determined surface tensions by an equation of state (3).

A number of techniques for evaluation of protein surface tension (γ_{pv}) have been described (4,5). Typical values near physiological pH are in the 65-71 ergs/cm^2 range. Surface tensions of supports (γ_{sv}) may be estimated by techniques such as sedimentation bed volume (6).

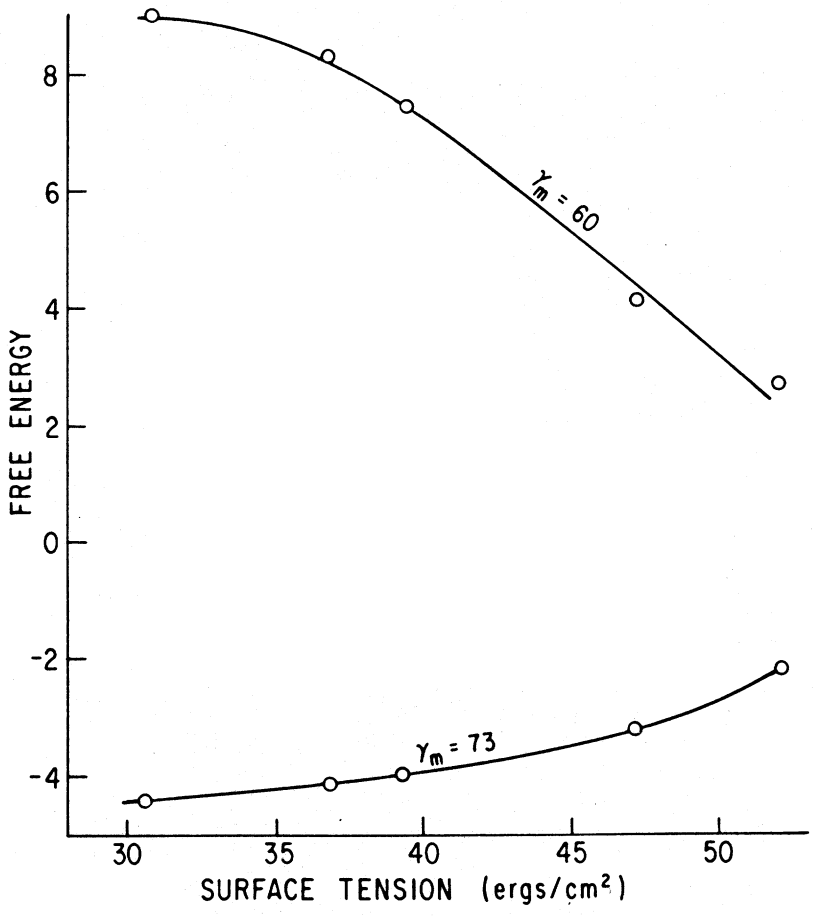
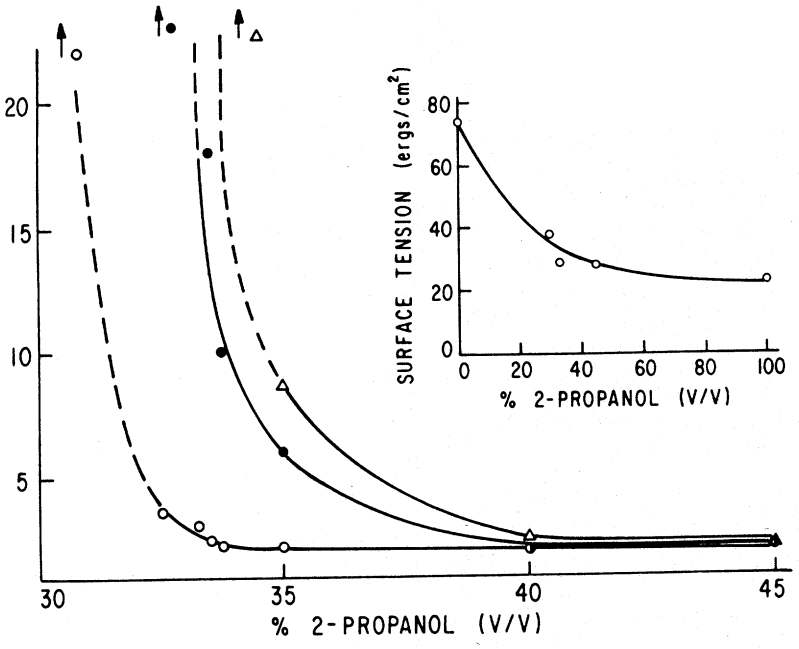


FIG. 7.2. Effect of packing on protein sorption. Modified silicas are, from left: n-hexyl, n-octadecyl, t-butyl, diol and diether. Surface tension of protein (BSA) taken as 70 ergs/cm^2, γ_{mv} = surface tensions of two mobile phases.

: effect of support surface tension on the sorption energy as ced from the aforementioned equation of state is deposited in e 7.2. The γ_{pv} was taken as that of bovine serum albumin (BSA) r physiological conditions (7). Values of γ_{mv} were chose to brack- at of γ_{pv}. The higher value is that of water, while the lower value l be obtained by the addition of ca. 10% alcohol to water and, as , resembles an HPLC mobile phase. Column supports represented n order of increasing γ_{sv}, hexyl–, octadecyl–, t-butyl–, deactivat- ether–, diol– and diether. Strategies for the chromatography of pro- with modified silicas are illustrated by Figure 7.2. Since sorption ored when $\gamma_{mv} > \gamma_{pv} > \gamma_{sv}$, elution of proteins may be induced by ction of γ_{mv}. Indeed, the literature (8) has shown (Fig. 7.3) that small changes in mobile phase composition (0.5% isopropanol) can ce elution of proteins at the column void volume which were not rved to elute at slightly higher buffer proportion. It is clear that would be sorbed strongly by aklylsilicas from water or buffer so- ns.



7.3. Relation of mobile phase surface tension to protein retention: (○) Bovine m albumin, (●) β-lactoglobulin, (△) hemaglobin. Mobile phase: 0.05 M phos- e buffer-isopropanol (pH 2.1). Column: octadecyl silica. (8).

The alkylsilica γ_{sv} are clustered in the 32-39 ergs/cm^2 range regardless of alkyl chain length or presence of a branched chain. Little difference in sorption of selectivities are expected. Inclusion of an aryl group could alter selectivity through interactions although γ_{sv} is in the same range.

When the organic moieties that are covalently bound to the silica surface have a large number of ether or polyol groups, the surface tension increases. In water or buffer–protein solutions then, strong sorption is not favored unless the salt concentration is very high because surface tension of water tends to increase with increasing salt concentration. The interaction energy becomes increasingly negative as γ_{mv} increases, all other factors being constant. This diminished tendency for proteins to sorb to such chemically modified silicas makes these packages especially suited for size-exclusion chromatography.

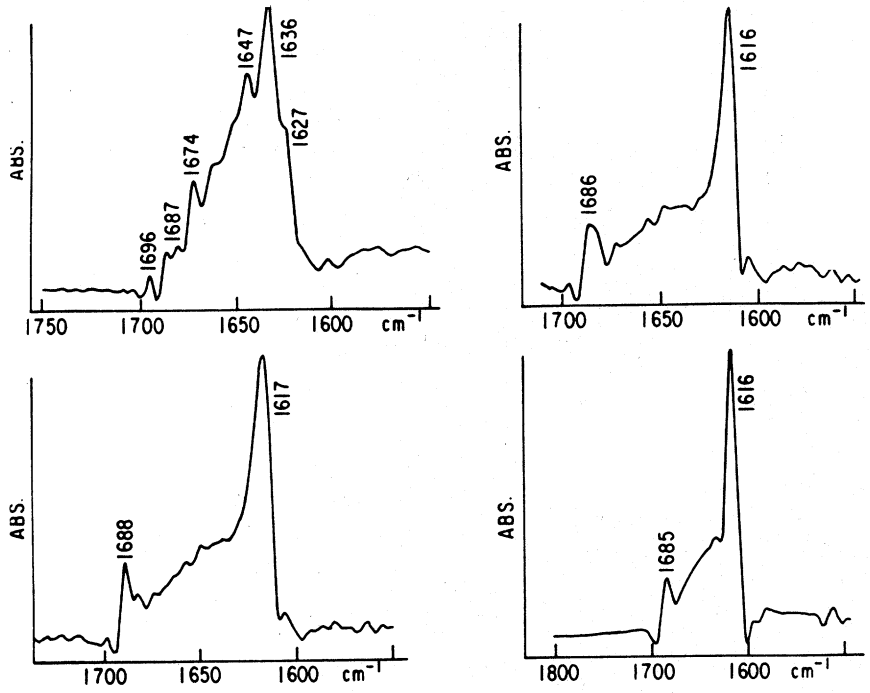
Mobile phase selection.

Typically, desorption or elution properties from reversed-phase supports is accomplished by reducing mobile phase surface tension with addition of alcohols or acetonitrile to acidic buffers. The eluents also contain phosphate or trifluoroacetic acid. These additives form ion-pairs with protein cationic sites and therefore influence the surface properties of proteins. Mobile phase surface tension decreases also, with temperature increase. Although net protein hydrophobicity (9) and protein size (10) are general indicators of relative retention in reversed phase chromatography, many anomalies are observed in the literature. These have been related to inherent conformational differences, conformational changes upon sorption and other factors. It must be noted, however, that Van der Waal interaction between hydrophilic groups and hydrophobes may be appreciable contributors to the total interaction energy (11). Nevertheless, since proteins have characteristic surface tensions and since very small changes in solvent composition effect large changes in retention (Fig. 7.3), carefully controlled concentration gradients are required for resolution of protein mixtures. Over the narrow elution range the capacity factor, k, at a mobile phase concentration is given by: (eq. 2) $\log k_l' = \log k_w'$-SE where E is the volume fraction of organic component, k_w' is the capacity factor in water and S is a solvent strength parameter that reflects properties of solvent and protein (12). When eq. 2 is determined for two proteins and combined with expressions to describe gradient time, conditions for resolving the proteins in mixtures may be deduced. In general, decreasing flow rate

d gradient steepness parameter will improve separation. Use of short lumns (<10 mm) give the same resolution of macromolecules (13) as ıger columns (<250 mm), and recoveries are improved.

duced structural changes.

ı important question to protein chemists is the effect of solvent, lumn and system components on structure and function of recovered oteins. Optical rotatory dispersion (ORD) (14), kinetic studies (15) and ıurier transform infrared spectroscopy (FT-IR) are yielding more ·finitive information. As shown above, copious quantities of organic ımponent are required to desorb most proteins from reversed phase pports. Figure 7.4a, b, c and d show a series of deconvolved FT-IR ectra of chymotrypsinogen A in buffer, in 40% isopropanol–buffer



IG. 7.4 Deconvolved FT-IR spectra of α-chymotrypsinogen. (a) D_2O, pD7; (b) 40% opropanol pD7; (c) 40% isopropanol pD2; (d) 40% isopropanol pD7, ATR spectra ' packing slurry. (6,7)

(16) and in contact with a reversed phase packing, respectively (17). The spectra exhibit differences in the amide 1 region. Changes in location and proportions of bands in this region are indicative of changes in secondary protein structure. The bands at 1615-1618 cm^{-1} in the alcohol continuing spectra indicate that the denatured form of this protein contains a large amount of a special kind (distorted) of β-strands. The introduction of solvent induces this structure while the act of sorption appears not to cause additional major conformational change of this protein. The effects of solvent and packing may not be a general one for proteins. For many proteins, the kinetics of conformational change may confound chromatographic observations. Papain, for example, yields two peaks when injected onto a reversed-phase column (15) and eluted with isopropanol/buffer. When the peak exhibiting enzymatic activity is collected and reinjected, two peaks are observed again indicating that the conformational change is rapid compared to column residence time. Therefore, factors such as gradient shape, mobile phase composition, column dimensions and temperature may influence peak proportions and/or yields of enzyme.

Effect of pore diameter.

As discussed earlier, the surface tensions of reversed-phase supports are comparable. Indeed, when static sorption experiments were performed with BSA dissolved in 2-propanol (40%)/0.05M phosphate at pH 2.1, the data were fitted by a single line as determined by correlation coefficients greater than 0.97 (18). The line was the linearized form of the Langmuir Equation so that adherence indicated that apparent binding capacities and desorption constants were the same for packings of widely different aklyl chains (C_8 and C_{18}) and pore diameters (10 nm and 50 nm). However, while the thermodynamic contributions to chromatography with such packings are similar, observed chromatograms may differ because of kinetic factors. A major factor in peak dispersion or spreading in liquid chromatography is the slow transfer of solute in the packings. This factor can be expressed by: $C=(k\ D_m) / [30(1+k)^2(D_p)]$, where k is capacity factor, D_m is the diffusitivity in free solution, D_p is the diffusitivity in the pore and C is the coefficient of the third term in Knox plate height equation (19). If all other parameters of the column (length, inside diameter, particle diameter, flow rate, nature of bound phase, etc.) are the same, then C and, therefore height equivalent to a theoretical plate, are inversely proportional

$)_p$. As shown in Table 7.1, D_p decreases significantly as the radius polymer approaches the diameter of a pore (20). Once inside a pore, ymer diffusion out of it is impeded. It is apparent that highest effi- ıcy is predicted when pore diameter is 3-10 times the polymer di- ːter. For most proteins, then, pore diameters greater than 300 Å predicated.

BLE 7.1
ative Diffusion of Dextran[a] (20)

Polymer Rad. / Pore Rad.	Rel. Diffusion
0.01	0.95
0.08	0.95
0.12	0.93
0.34	0.43
0.49	0.22
1.00	0.04

[a]Rad. Dex. = 60 Å, D = 4 x 10^{-7} cm^2/s

rfactant mediated chromatography.

the earlier discussions, desorption of proteins from reversed-phase ːkings was accomplished by the addition of copious quantities of or- ıic solvent to buffer in order to lower surface tension of the mobile ıse. Use of non-ionic surfactants, which are known to perturb pro- n structure to a lesser degree than ionic ones (21) (22), have been plored as alternatives for lowering surface tension of mobile phase. primary effect, though, is the sorption of the surfactants to the drocarbonaceous packing. When micellar concentrations of surfac- ıt are used, chromatography of proteins is mediated through com- ːx equilibria (23). This approach, however, does have utility for protein ɔarations.

ıdrophobic interaction chromatography.

ıother approach to reducing energy of interaction of protein and cking is through the use of packing with higher surface tension rela- e to water. This can be done by creating polyether or polyamide func-

tionality over the silica surface then carrying out further chemical modification so that these hydrophillic surfaces are lightly substituted with hydrocarbon groups. Examples are shown schematically in Figure 7.5. These types of modified silicas have been labeled "hydrophobic interaction" packings because they mimic the carbohydrate gel-based materials that have been used by biochemists for some time (24). The behavior of four proteins on packings where the principal hydrophobic group was controlled chemically to occupy a varying fraction of possible sites on the bound polymeric layer is shown in Figure 7.6 (25). Chromatography was carried out at pH 7 (0.01 M phosphate buffer) with a gradient that decreased from 1 M sodium sulfate to just buffer. At low coverage, surface tensions of packing approach those on the right side of Figure 7.2 so that mobile phase tension is increased by salt ad-

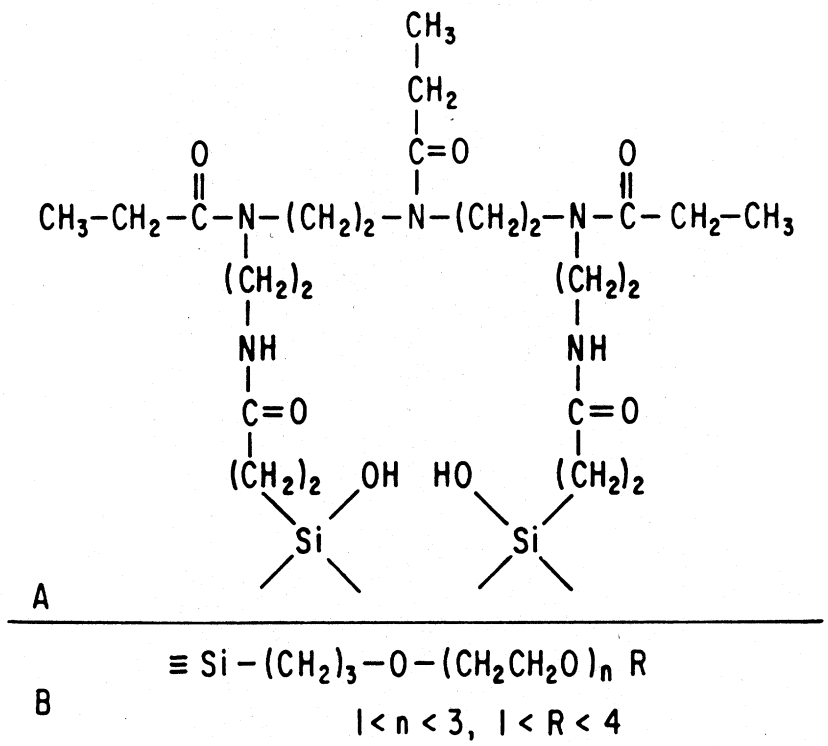
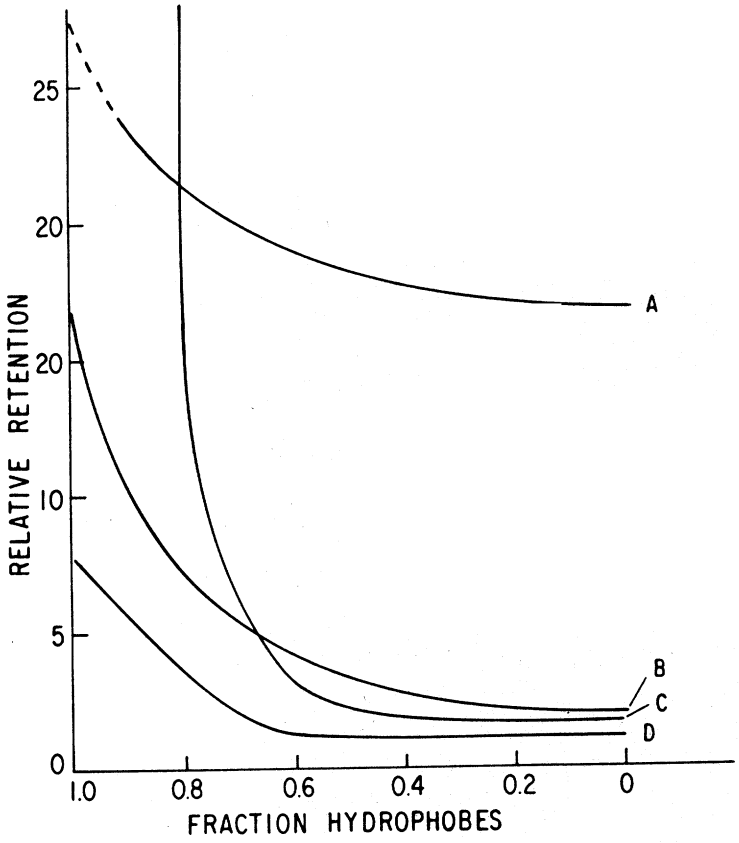


FIG. 7.5. Schematic of lightly hydrophobic surfaces.

n to promote sorption. Elution proceeds as mobile phase surface ion is reduced. As coverage exceeds about 60 percent, retention mes dramatically longer as would be predicted from analogy to re 7.2. Exposure of more protein hydrophobic groups through ges in conformation are not necessary to explain stronger sorp- onto reversed-phase supports than onto "hydrophobic interaction" . Only reductions in packing surface energy need to be considered, ough the sharp inflection of the BSA curve suggests that some ge may occur. As discussed earlier, addition of alcohol and/or lower-



7.6. Relative retention of some proteins on packings of varying hydrophobici- (a) α-Chymotrypsinogen; (b) ovalbumin; (c) bovine serum albumin; (d) uclease. Linear gradient from 1M Na_2SO_4 in 10 mM phosphate buffer (pH 7) to M phosphate buffer (ph 7). (25)

ing pH may induce conformational changes that enhance sorption. Surface tension of BSA was reduced from 70 ergs/cm^2 in saline to 35/ ergs/cm^2 in alcohol/buffer (8), for example.

It is clear that these lightly substituted packings will be used increasingly where separations of larger proteins are required and when active enzymes need to be recovered.

Ion-exchange chromatography.

Up to this point, discussed have been protein separations on packings that have zero or little surface charge so that sorption does not involve coulombic forces. The use of ionic carbohydrate-based polymers have been widely used by protein chemists for ion [illegible]

"Methods for Protein Analysis"

The following two equations appear incorrectly on page 98:

$$|Z|(P^{\pm 1} + El^{\pm 1}) + A^z \rightleftharpoons (ZP^{\pm 1} + A^z) + |Z|\ EL^{\pm 1} \quad (26).$$

$$K^c = \frac{K_{EL}\,[EL]_P^Z}{[EL]_m^z \left(1 + \dfrac{K_{eq}}{[H^+]_m^Z}\right)}$$

[illegible] loped for anionic solutes.

While proteins, because of their complex and heterogeneous nature, do not behave as idealized solutes in chromatographic systems, some generalizations may be made from the expression. The number of exchanging sites, Z, can be large for proteins, and since $[H^+]$ is raised to the Z power, small decreases in pH result in large increases in K^c. Similarly, large increases in mobile phase ionic strength result in greatly diminished K^c. K^c is related to retention through the expression, $k = K^c (V_s/V_m)$ where k is chromatographic capacity factor and (V_s/V_m), the solvent ratio, is assumed to be constant for a particular system under

he range of conditions used. Increase of NH_4Cl molarity by only 0.005 caused ribonuclease to be eluted at the column void volume where it had been sorbed totally to a cation ion-exchange packing at lower concentration. Thus, the phenomenon of a multisite binding is observed in ion–exchange as well as reversed phase chromatography. The number of interacting sites (Z) was determined chromatographically for several proteins found to correlate with charge on the protein surface (27). Denatured chymotrypsinogen–A, however, elutes earlier than the native form although the number of binding sites was higher (28). Apparently, weaker interactions occurred in the former.

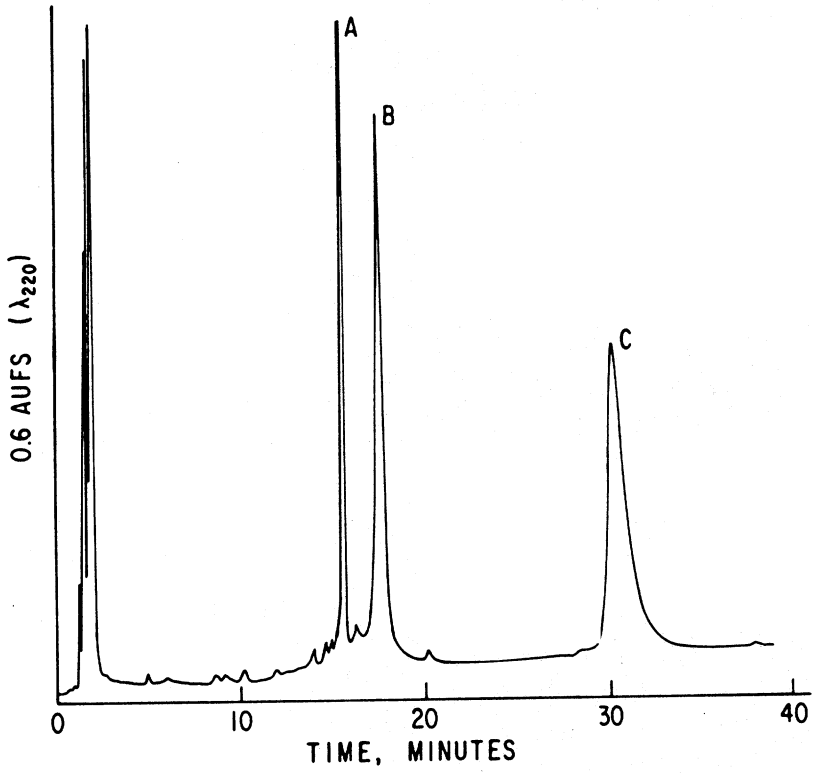


FIG. 7.7. Cation-exchange chromatography of proteins. (a) Ribonuclease; (b) α-chymotrypsin; (c) lysozyme. Mobile phase: linear gradient from 10 mM phosphate bufer (pH 7) to 200 mM phosphate buffer + 1M NaCl (pH 7). (29)

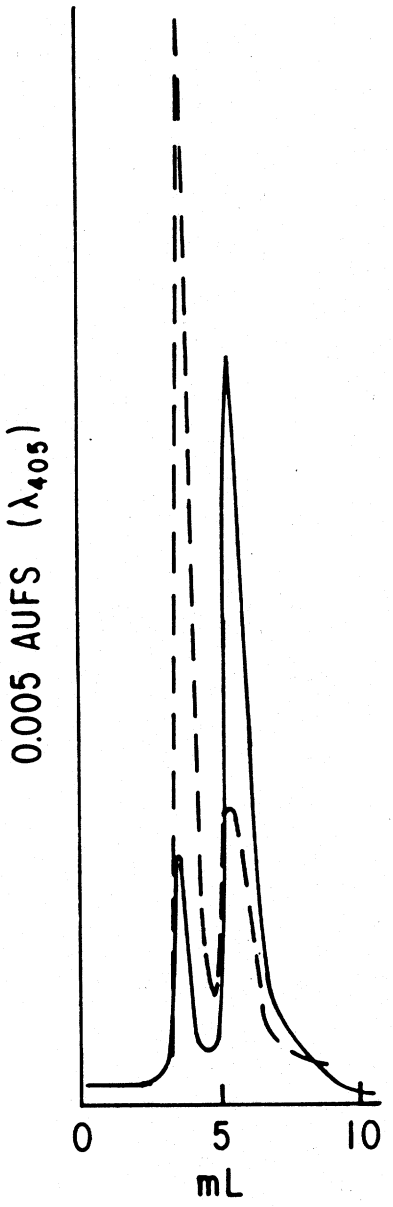
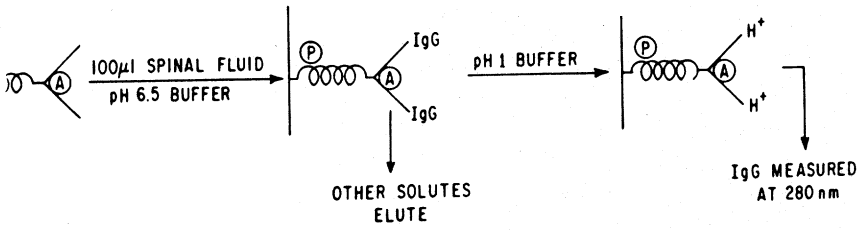


FIG. 7.8 Anion-exchange separation of myoglobin forms (–) freshly prepared from muscle, (– – –) after 2 hrs. (30)

examples of high performance ion-exchange separations of pro- are given in Figures 7.7 and 7.8. The first demonstrates the sepa- of several basic proteins by a cation-exchange column with hate buffer, pH 7, as mobile phase (29). Elution was achieved gh a gradient of increasing salt concentration. Ribonuclease and otrypsin have iso-ionic points of 9.3 and 8.8, respectively, while of lysozyme is 11. The second example shows the separation of -exchange of muscle myoglobin immediately after isolation and 2 hours' incubation at 37 C (30). Concomitant examination by exclusion chromatography demonstrated that both components imilar molecular sizes and both exhibited myoglobin functionali- measured by peroxidase activity reactivity. It was concluded, then, anion-exchange chromatography revealed subtle charge differ- . It is clear that highly selective separations may be carried out ion-exchange chromatography and that elution can be controlled organic solvent-free buffer solutions. The use of wide-pore E-silica cartridges and DEAE-cellulose disks have been used re- y to indicate the presence of 50Y protein in meat protein isolates

ity chromatography.



7.9 Schematic for determination of immunoglobulins by high performance af- chromatography. (P) protein (covalently bound); (a) antibody (bound by cross- g). (33)

ity columns offer degrees of specificity for single analytes that often not achievable by other modes of chromatography (32). To eve this specificity, diol or other polar modified silica is reacted fur- to attach moieties such as imidazole that can form covalent link- with free amino groups on proteins, enzyme inhibitors or odies. The analyte interacts with these moieties under conditions favor its selective binding. The analyte is then displaced by a change

in conditions. Analyses that are time consuming, difficult because of the complexity of the mixture, or require detection of low levels, are facilitated by affinity chromatography. Common techniques for immunoglobins (IgG), for example, may take up to 24 hours. When IgG antibody is immobilized, it forms the basis for analysis in one hour (33). The procedure is shown schematically in Figure 7.9.

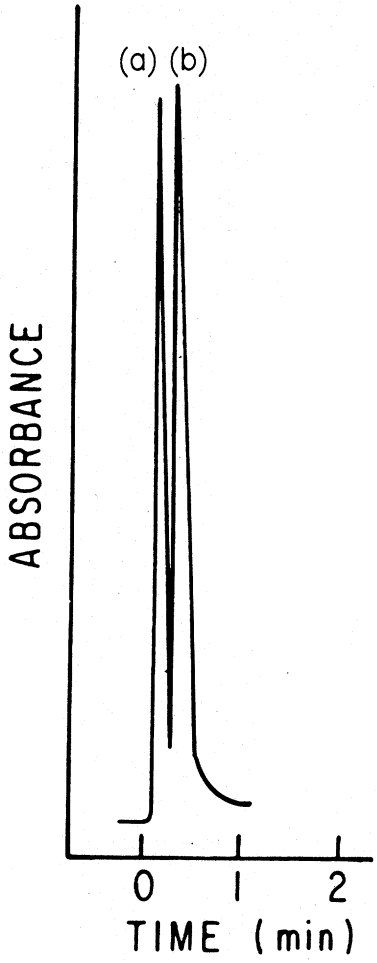


FIG. 7.10 High performance affinity chromatography. (a) inactive trypsin; (b) active trypsin. Column: immobilized soybean inhibitor (6.3 X 4.1 mm). Mobile phase: step gradient from pH 7 to pH 2.5, 100 mM phosphate buffer. (34)

Loss in trypsin activity is measured conveniently with the use of ɔlumns in which immobilized trypsin inhibitor was packed. The mixıre is passed through the column at pH 7. Inactive trypsin elutes at ıis pH, and active enzyme is retained. Its desorption is affected by ·wering the pH to 2 (Fig. 7.10). The proportion of native and deactiıted enzyme is easily measured from heights or areas. While general ɔrption may be reduced by varying the length of organic groups atıching the specific moiety to silica surface, it may also be reduced by ıe use of minicolumns. These have the additional advantage of requirıg less packing which tends to be expensive. Increased use of this approach for analysis of food proteins is imminent.

'ressure-packed gel columns.

. major disadvantage of columns packed with modified silicas is their ost. This may be particularly critical when they are used for analyses f protein mixtures isolated from animal tissue, leaves or seeds because ıe isolates often contain other labile biopolymers. These materials and ıeir reaction products contribute to shortened column life. For such pplications, the use of gels for size exclusion chromatography and modied gels provide an alternative. Contrary to general belief, gels may e packed into columns under moderate pressures (35) and may be used ontinuously for about 6 months. A four column set, with a molecular /eight operating range of 0.2–700 kilodaltons, provided excellent resoıtion when operated so that separation was completed in about 2 ours. With automated operation, a number of isolates from plants were ·rocessed in a day, and new information was obtained on the composiion of forage protein isolates (36) as well as on the chemistry of ensilng (37). Recently, reduced particle diameter gels in which rigidity was ncreased through cross-linking were introduced (38). These studies ılso verified previous observations that pressure compression of the ;el bed increased resolution presumably by increasing the ratio of paricle inner volume to void volume (39). The use of gels, therefore, should ıot be overlooked and may be preferred in some cases.

:ffect of other system components on protein recovery.

'he extrusion of proteins through stainless steel capillary tubing and :olumn-end frits may induce conformational changes that contribute o irreversible sorption to these components of the HPLC system. Someimes the hydrodynamic flow patterns within the capillary are altered

markedly, promoting the formation of metastable aggregates of protein precipitate (40). These may dissociate slowly and contribute to erratic results in subsequent experiments. In a similar manner, release of protein sorbed to frits is dependent on solvent volume pumped between injections and, therefore, would affect the reproducibility of chromatography. As much as 33% of injected lysozyme has been found to be sorbed to stainless steel frits (41). It is clear that these effects must be considered in the design of protein separations by chromatography.

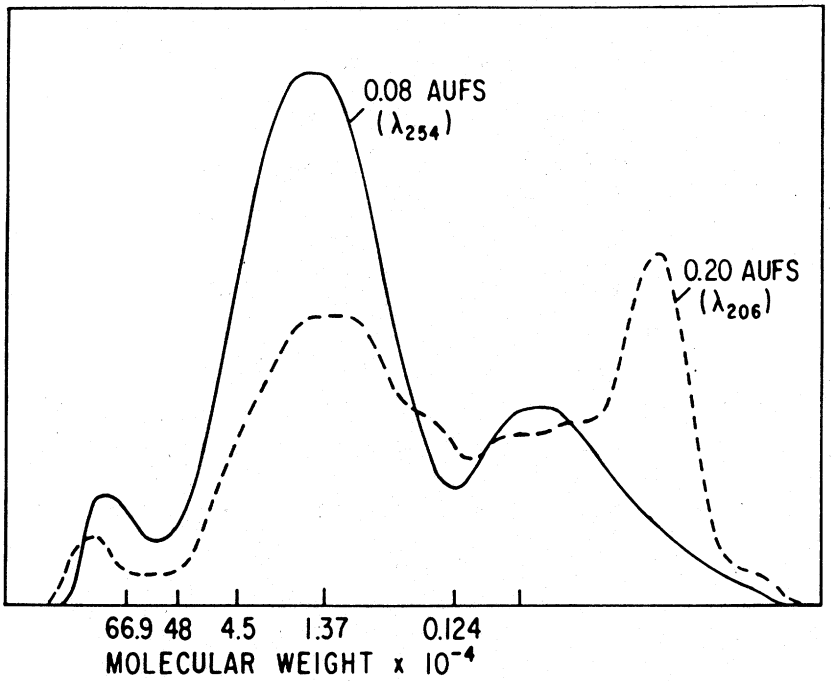


FIG. 7.11 Pressure-packed gel chromatogram of grain sorghum glutelin. (40)

Protein detection.

Detection of proteins as they elute from the chromatographic column is accomplished most often by ultraviolet (UV) detection at 280 nm. Monitoring at 206 nm provides general concentration detection that is essentially independent of amino acid composition of protein. Combination of the two can provide additional information about separat-

ed species. Figure 7.11 not only illustrates the use of pressure-packed gel columns, but demonstrates advantage of dual-wavelength detection (42). Here, tannin-glutelin complexes from grain sorghum are chromatographed. The shorter wavelength monitors amide absorption and, therefore concentration, while the longer determines distribution of aromatic (phenolic) species. Flourimetry is often 10 to 100 times more sensitive for proteins than UV and, with appropriate filtering, can be selective. Since spurious peaks are encountered often when extracts from biological matrices are injected into liquid chromatographs, confirmation of protein peaks is essential. Performance of colorimetric reactions or other tests is facilitated by on-line post-column reactors. Contributions to band broadening by various reactor types were investigated and are summarized in Table 7.2 for a standard reaction (42). When reaction times were 1-2 minutes, all of the devices appeared suitable for use in the typical HPLC system (30 X 0.4 cm column, K'ξ2). None were useful when longer reaction times were necessary. Nevertheless, such systems are being used more for specific detection of analytes.

TABLE 7.2
Band Broadening of Reactors[a] (42)

	sec
COILED TUBULAR, 25 m x 0.25 mm	2.3
COILED TUBULAR, 6 m x 0.5 mm	4.5
KNITTED TUBULAR, 6 m x 0.5 mm	4.0
PACKED BED, 20 cm x 4.6 mm, d_p = 40 m	1.7
PACKED BED, 20 cm x 4.6 mm, d_p = 17 m	1.2
SEGMENTED FLOW, 2.0 mm I.D. + DEBUBBLER	2.2
SEGMENTED FLOW, 1.1 mm I.D. + DEBUBBLER	1.0

[a]OPERATED UNDER STANDARD CONDITIONS

Sample preparation.

Improved methods for isolating proteins from food and/or biological matrices is a critical step in the analytical process. Simplification of mixtures through fractionation of buffer extracts by ammonium sulfate precipitation schemes before chromatography is common. Other approaches involve successive solubilization steps by addition of extractants such as sodium dodecylsulfate (SDS) to free protein according to the extent to which they are matrix bound. Differences in protein size distributions that were obtained from grass leaf protein isolates are

shown in Table 7:3. Trends in molecular weight with change in SDS concentration were explained by postulating that the surfactant breaks up cholorplasts and dissolves chloroplastic proteins, while in the absence of SDS, only cytoplasmic proteins are extracted.

TABLE 7.3
Molecular Weights From Gel Chromatography (34)

%SDS (W/V)	206 nm $Ma_1 X 10^{-3}$	254 nm $Ma_1 X 10^{-3}$
0	21.2 ± 1.2	5.2 ± 6.7
0.2	10.5 ± 2.4	4.6 ± 2.7
0.5	5.4 ± 1.0	6.3 ± 1.7
1.0	9.9 ± 3.4	13.7 ± 5.0

Modified silicas having functionalities similar to those used in HPLC but with larger particle sizes and smaller surface areas are being used increasingly for pre-fractionation and sample clean-up. Such materials are less expensive than HPLC packings and are frequently employed in disposable gravity flow columns or cartridges that can be fitted to syringe fittings where they function as mini extractors. Sorbed analytes can be desorbed for further study by appropriate solvent change.

HPLC, aided by greater understanding of retention mechanisms and availability of improved column packings, has greatly reduced the time required for protein analysis. Use of multi-wavelength detectors and post-column, on-line derivativization facilitates quantitation and specificity. As the following chapters indicate, HPLC is revolutionizing the analytical chemistry of proteins.

Acknowledgments

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ing pH may induce conformational changes that enhance sorption. Surface tension of BSA was reduced from 70 ergs/cm^2 in saline to 35/ ergs/cm^2 in alcohol/buffer (8), for example.

It is clear that these lightly substituted packings will be used increasingly where separations of larger proteins are required and when active enzymes need to be recovered.

Ion-exchange chromatography.

Up to this point, discussed have been protein separations on packings that have zero or little surface charge so that sorption does not involve coulombic forces. The use of ionic carbohydrate-based polymers have been widely used by protein chemists for ion-[illegible]

"Methods for Protein Analysis"

The following two equations appear incorrectly on page 98:

$$|Z|(P^{\pm 1} + El^{\pm 1}) + A^z \rightleftharpoons (ZP^{\pm 1} + A^z) + |Z|\ EL^{\pm 1} \quad (26).$$

$$K^c = \frac{K_{EL}\,[EL]_P^Z}{[EL]_m^z\left(1 + \dfrac{K_{eq}}{[H^+]_m^Z}\right)}$$

loped for anionic solutes.

While proteins, because of their complex and heterogeneous nature, do not behave as idealized solutes in chromatographic systems, some generalizations may be made from the expression. The number of exchanging sites, Z, can be large for proteins, and since $[H^+]$ is raised to the Z power, small decreases in pH result in large increases in K^c. Similarly, large increases in mobile phase ionic strength result in greatly diminished K^c. K^c is related to retention through the expression, $k = K^c (V_s/V_m)$ where k is chromatographic capacity factor and (V_s/V_m), the solvent ratio, is assumed to be constant for a particular system under

he range of conditions used. Increase of NH_4Cl molarity by only 0.005 caused ribonuclease to be eluted at the column void volume where it had been sorbed totally to a cation ion-exchange packing at lower concentration. Thus, the phenomenon of a multisite binding is observed in ion–exchange as well as reversed phase chromatography. The number of interacting sites (Z) was determined chromatographically for several proteins found to correlate with charge on the protein surface (27). Denatured chymotrypsinogen–A, however, elutes earlier than the native form although the number of binding sites was higher (28). Apparently, weaker interactions occurred in the former.

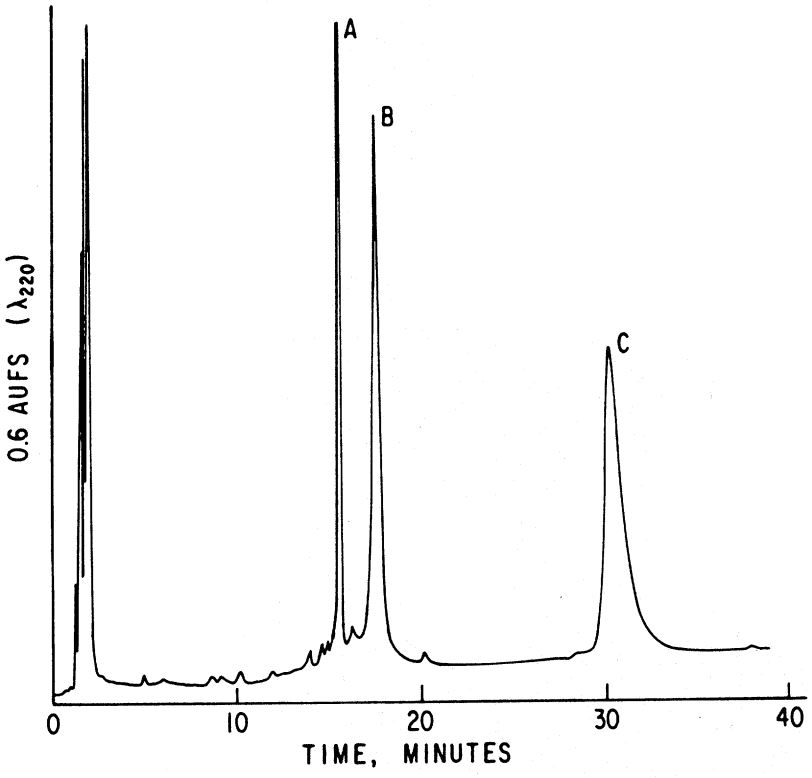


FIG. 7.7. Cation-exchange chromatography of proteins. (a) Ribonuclease; (b) α-chymotrypsin; (c) lysozyme. Mobile phase: linear gradient from 10 mM phosphate bufer (pH 7) to 200 mM phosphate buffer + 1M NaCl (pH 7). (29)

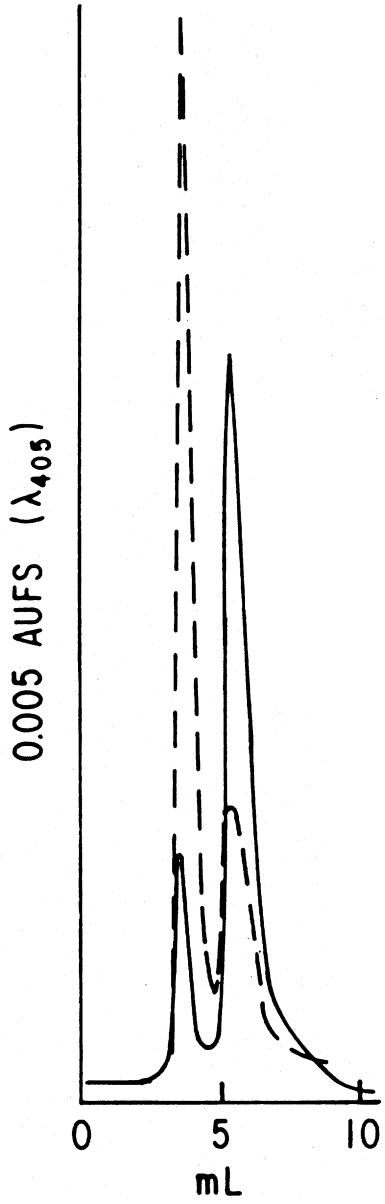
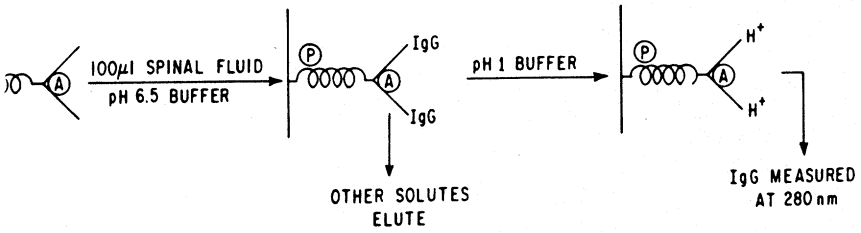


FIG. 7.8 Anion-exchange separation of myoglobin forms (–) freshly prepared from muscle, (– – –) after 2 hrs. (30)

examples of high performance ion-exchange separations of pro- are given in Figures 7.7 and 7.8. The first demonstrates the sepa- of several basic proteins by a cation-exchange column with hate buffer, pH 7, as mobile phase (29). Elution was achieved gh a gradient of increasing salt concentration. Ribonuclease and otrypsin have iso-ionic points of 9.3 and 8.8, respectively, while of lysozyme is 11. The second example shows the separation of -exchange of muscle myoglobin immediately after isolation and 2 hours' incubation at 37 C (30). Concomitant examination by exclusion chromatography demonstrated that both components imilar molecular sizes and both exhibited myoglobin functionali- measured by peroxidase activity reactivity. It was concluded, then, anion-exchange chromatography revealed subtle charge differ-. It is clear that highly selective separations may be carried out ion-exchange chromatography and that elution can be controlled organic solvent-free buffer solutions. The use of wide-pore E-silica cartridges and DEAE-cellulose disks have been used re- y to indicate the presence of 50Y protein in meat protein isolates

ity chromatography.



7.9 Schematic for determination of immunoglobulins by high performance af- chromatography. (P) protein (covalently bound); (a) antibody (bound by cross- g). (33)

ity columns offer degrees of specificity for single analytes that often not achievable by other modes of chromatography (32). To eve this specificity, diol or other polar modified silica is reacted fur- to attach moieties such as imidazole that can form covalent link- with free amino groups on proteins, enzyme inhibitors or odies. The analyte interacts with these moieties under conditions favor its selective binding. The analyte is then displaced by a change

in conditions. Analyses that are time consuming, difficult because of the complexity of the mixture, or require detection of low levels, are facilitated by affinity chromatography. Common techniques for immunoglobins (IgG), for example, may take up to 24 hours. When IgG antibody is immobilized, it forms the basis for analysis in one hour (33). The procedure is shown schematically in Figure 7.9.

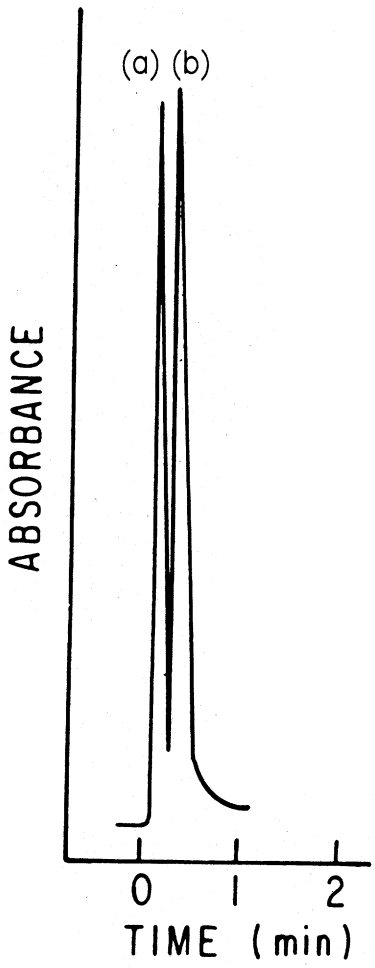


FIG. 7.10 High performance affinity chromatography. (a) inactive trypsin; (b) active trypsin. Column: immobilized soybean inhibitor (6.3 X 4.1 mm). Mobile phase: step gradient from pH 7 to pH 2.5, 100 mM phosphate buffer. (34)

Loss in trypsin activity is measured conveniently with the use of olumns in which immobilized trypsin inhibitor was packed. The mixre is passed through the column at pH 7. Inactive trypsin elutes at is pH, and active enzyme is retained. Its desorption is affected by wering the pH to 2 (Fig. 7.10). The proportion of native and deactited enzyme is easily measured from heights or areas. While general rption may be reduced by varying the length of organic groups atching the specific moiety to silica surface, it may also be reduced by e use of minicolumns. These have the additional advantage of requirg less packing which tends to be expensive. Increased use of this approach for analysis of food proteins is imminent.

ressure-packed gel columns.

major disadvantage of columns packed with modified silicas is their ost. This may be particularly critical when they are used for analyses f protein mixtures isolated from animal tissue, leaves or seeds because e isolates often contain other labile biopolymers. These materials and eir reaction products contribute to shortened column life. For such pplications, the use of gels for size exclusion chromatography and modied gels provide an alternative. Contrary to general belief, gels may e packed into columns under moderate pressures (35) and may be used ontinuously for about 6 months. A four column set, with a molecular eight operating range of 0.2–700 kilodaltons, provided excellent resotion when operated so that separation was completed in about 2 ours. With automated operation, a number of isolates from plants were rocessed in a day, and new information was obtained on the composiion of forage protein isolates (36) as well as on the chemistry of ensilng (37). Recently, reduced particle diameter gels in which rigidity was ncreased through cross-linking were introduced (38). These studies lso verified previous observations that pressure compression of the el bed increased resolution presumably by increasing the ratio of paricle inner volume to void volume (39). The use of gels, therefore, should ot be overlooked and may be preferred in some cases.

ffect of other system components on protein recovery

he extrusion of proteins through stainless steel capillary tubing and olumn-end frits may induce conformational changes that contribute o irreversible sorption to these components of the HPLC system. Someimes the hydrodynamic flow patterns within the capillary are altered